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Genomic Comparison of Virulent *Rickettsia rickettsii* Sheila Smith and Avirulent *Rickettsia rickettsii* Iowa▽

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Rickettsia rickettsii **is an obligate intracellular pathogen that is the causative agent of Rocky Mountain spotted fever. To identify genes involved in the virulence of *R. rickettsii*, the genome of an avirulent strain, *R. rickettsii* Iowa, was sequenced and compared to the genome of the virulent strain *R. rickettsii* Sheila Smith. *R. rickettsii* Iowa is avirulent in a guinea pig model of infection and displays altered plaque morphology with decreased lysis of infected host cells. Comparison of the two genomes revealed that *R. rickettsii* Iowa and *R. rickettsii* Sheila Smith share a high degree of sequence identity. A whole-genome alignment comparing *R. rickettsii* Iowa to *R. rickettsii* Sheila Smith revealed a total of 143 deletions for the two strains. A subsequent single-nucleotide polymorphism (SNP) analysis comparing Iowa to Sheila Smith revealed 492 SNPs for the two genomes. One of the deletions in *R. rickettsii* Iowa truncates *rompA*, encoding a major surface antigen (rickettsial outer membrane protein A [rOmpA]) and member of the autotransporter family, 660 bp from the start of translation. Immunoblotting and immunofluorescence confirmed the absence of rOmpA from *R. rickettsii* Iowa. In addition, *R. rickettsii* Iowa is defective in the processing of rOmpB, an autotransporter and also a major surface antigen of spotted fever group rickettsiae. Disruption of *rompA* and the defect in rOmpB processing are most likely factors that contribute to the avirulence of *R. rickettsii* Iowa. Genomic differences between the two strains do not significantly alter gene expression as analysis of microarrays revealed only four differences in gene expression between *R. rickettsii* Iowa and *R. rickettsii* strain R. Although *R. rickettsii* Iowa does not cause apparent disease, infection of guinea pigs with this strain confers protection against subsequent challenge with the virulent strain *R. rickettsii* Sheila Smith.**

Rickettsia rickettsii is a member of the spotted fever group of rickettsiae and the etiologic agent of Rocky Mountain spotted fever (RMSF). *R. rickettsii* is a small obligate intracellular gram-negative organism that is maintained in its tick host through transovarial transmission (17, 31). Infection with *R. rickettsii* occurs through the bite of an infected tick. Once the organism gains access to the host, it is able to replicate within the host vascular endothelial cells and spread from cell to cell by polymerizing host cell actin (20). Damage to vascular endothelial cells by *R. rickettsii* leads to increased vascular permeability and leakage of fluid into the interstices, causing the characteristic rash observed in RMSF (19). Infection with *R. rickettsii* results in a severe and potentially life-threatening disease if it is not diagnosed and treated properly. While much is known about the progression of the disease, the molecular mechanisms involved in the pathogenesis of RMSF are poorly understood.

Rickettsiae are separated into two groups: the spotted fever group and the typhus group. The genomes of several rickettsiae have recently been completed and have provided an abundance of genomic information; these genomes include those of *R. bellii* RML369-C (27), *R. conorii* Malish 7 (26), *R. felis* URRWXCal2 (28), *R. prowazekii* Madrid E (6), *R. rickettsii* Sheila Smith (GenBank accession no. AADJ01000001), *R. sibirica* (GenBank accession no. AABW01000001), and *R. typhi* Wilmington (25). The availability of genomic sequences allows comparisons between the two groups (25) and between virulent and avirulent strains of rickettsiae (13).

R. rickettsii Iowa was obtained from guinea pigs inoculated with a *Dermacentor variabilis* suspension (8). Interestingly, *R. rickettsii* Iowa displayed various degrees of virulence in the guinea pig infection model during passage in eggs (8). Early passages showed mild virulence, but subsequently this strain became highly virulent before eventually displaying an avirulent phenotype. Analysis of a high-egg-passage clone demonstrated that the strain was deficient in the ability to lyse Vero cells, forming indistinct plaques compared to the clear plaques observed for *R. rickettsii* strain R (18). Is was also found that *R. rickettsii* Iowa was defective in processing rickettsial outer membrane protein B (rOmpB) from its 168-kDa precursor into its 120- and 32-kDa forms (18). It has yet to be determined if the inability of *R. rickettsii* Iowa to lyse Vero cells and cause infection in guinea pigs is the result of defective rOmpB processing, some other mutation, or a combination of these two factors.

The lack of good genetic tools for rickettsiae has made the identification of virulence genes difficult. A number of studies have looked at genetic, antigenic, and phenotypic differences between unique *R. rickettsii* strains (1, 3, 4, 12). However, the

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complete genomes of virulent and avirulent strains of *R. rickettsii* have yet to be compared.

Recently, the genomic sequence of the virulent strain *R. rickettsii* Sheila Smith was completed (GenBank accession no. AADJ01000001). To identify genes potentially involved in the virulence of *R. rickettsii*, we determined the genomic sequence of the cloned avirulent strain *R. rickettsii* Iowa (GenBank accession no. CP000766) and compared it to the *R. rickettsii* Sheila Smith genomic sequence. Here we describe genomic and expression differences that may contribute to the avirulence of *R. rickettsii* Iowa.

MATERIALS AND METHODS

Rickettsiae. *R. rickettsii* strain R, *R. rickettsii* Sheila Smith, and *R. rickettsii* Iowa (8) were propagated in Vero cells using M199 medium and were purified by Renografin density gradient centrifugation (33).

Genomic DNA purification. To isolate *R. rickettsii* Iowa genomic DNA, purified *R. rickettsii* Iowa was first lysed by incubation in 50 mM Tris-HCl (pH 8.0), 50 mM EDTA, 1% sodium dodecyl sulfate, 10 mM dithiothreitol, 0.1 mg/ml proteinase K for 2 h at 60°C. After 2 h, 1 volume of chloroform-isoamyl alcohol was added, and the mixture was centrifuged for 3 min at 20,000 × *g*. The aqueous phase was removed and subjected to another round of chloroform-isoamyl alcohol extraction. DNA was precipitated with 0.1 volume of 3 M sodium acetate (pH 5.0) plus 0.6 volume of isopropanol and resuspended in Tris-EDTA (pH 8.0).

Genome sequencing, annotation, and alignment. The genome of *R. rickettsii* Iowa (GenBank accession no. CP000766) was sequenced by Integrated Genomics, Inc. using standard sequencing procedures (10, 21, 23). For regions of the genome with low sequence quality, directed sequencing was performed to increase the minimum consensus base quality to an average of Q40 (99.99% accuracy of base call) throughout. Manual efforts and proprietary software (Integrated Genomics) were used to identify open reading frame (ORFs) in the genome of *R. rickettsii* Iowa, and the ORFs were then entered into the ERGO bioinformatics suite (Integrated Genomics) for final annotation (29). GC skew was calculated by determining $(C - G)/(G + C)$ with a 20-kb sliding window moving in 500-bp incremental steps. The G+C content was calculated using 2- and 20-kb sliding windows compared to the total G+C content of the entire chromosome.

The similarity threshold was set at $10e^{-10}$ for the conserved-versus-unique ORF comparison of *R. rickettsii* Sheila Smith and *R. rickettsii* Iowa. Annotation of the *R. rickettsii* Iowa genome produced 245 ORFs that were not called or were absent from *R. rickettsii* Sheila Smith. A focused ORF-to-ORF comparison and a corresponding DNA-to-DNA comparison were performed for the *R. rickettsii* Iowa annotation and the unfinished *R. rickettsii* Sheila Smith genome annotation. Based on this analysis, 223 of the 245 ORFs were found to be present in *R. rickettsii* Sheila Smith. These ORFs were removed from *R. rickettsii* Iowa during our comparison in order to obtain a more accurate assessment of unique genes versus conserved genes. However, the annotation files submitted to GenBank contain these 223 ORFs.

A whole-genome alignment was performed using MAUVE (9) software according to the manufacturer's instructions. All other DNA and protein alignments were performed using the ERGO bioinformatics suite.

SNP identification protocol. To identify single-nucleotide polymorphisms (SNPs), all ORFs, RNAs, and intergenic regions from *R. rickettsii* Sheila Smith and *R. rickettsii* Iowa were clustered into these groups. The DNA types were further filtered using a similarity cutoff of 80% of the bases between the two genomes of the same type. If the overall lengths of features differed by less than 10%, the features were considered clustered and were used for calculation of SNPs. Features whose lengths differed by more than 10% were analyzed manually. All clusters of each feature type were aligned using ClustalW, and SNPs were assigned where the aligned sequences had a change in a nucleotide at a specific location in the alignment.

Multilocus sequence alignment. DNA coding sequences of the *glt*, *gyrB*, *ompB*, *recA*, and *sca4* genes were collected from the following genomes (genome accession numbers are indicated in parentheses): *R. akari* (NZ_AAFE00000000), *R. felis* (CP000053.1), *R. conorii* (AE006914.1), *R. sibirica* (NZ_AABW00000000), *R. rickettsii* (NZ_AADJ00000000), *Rickettsia prowazekii* (AJ235269.1), *R. typhi* (AE017197.1), *R. canadensis* (NZ_AAFF00000000), and *R. bellii* (NZ_AARC00000000). Gene sequences were concatenated 5′ to 3′ in the following order: *glt*, *gyrB*, *ompB*, *recA*, *sca4*. Individual genes and concatenated sequences at the nucleotide and deduced amino acid sequence levels were analyzed with MacVector software (version 6.0; Oxford Molecular). Individual and concatenated DNA sequences were first aligned with the Clustal V program in the Lasergene software package (Dnastar). The alignments were transferred into the MacClade (Sinauer Associates) program for manual correction. MacClade output files were opened in PAUP (Sinauer Associates), and maximum-likelihood neighbor-joining trees were generated. Trees were rooted by making the outgroup (*R. bellii*) paraphyletic with respect to the ingroup. The robustness of clade designations was tested with a full heuristic search and 10,000 bootstrap replicates.

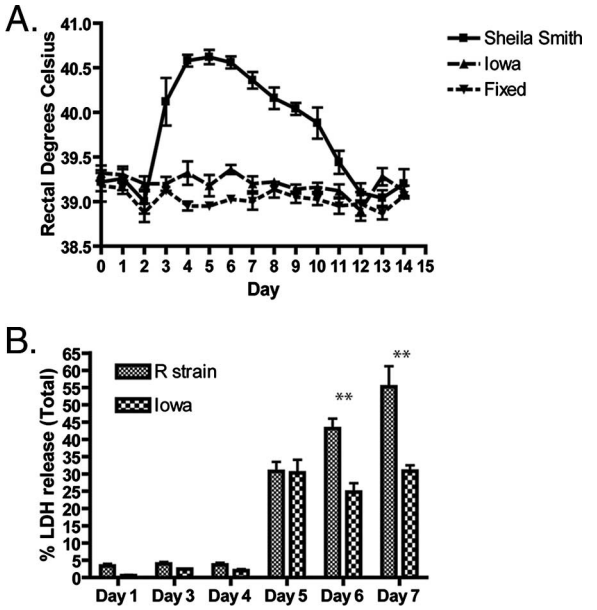

FIG. 1. *R. rickettsii* Iowa virulence and the ability of *R. rickettsii* Iowa to lyse Vero cells are attenuated. (A) Female Hartley guinea pigs were infected with 1,000 PFU of *R. rickettsii* Sheila Smith or *R. rickettsii* Iowa or inoculated with paraformaldehyde-fixed rickettsiae (Fixed), and their temperatures were monitored for 14 days postinfection. (B) Monolayers of Vero cells grown in 96-well plates were infected with either *R. rickettsii* strain R or *R. rickettsii* Iowa, and the percentages of LDH released into the media were determined on days 1, 3, 4, 5, 6, and 7 postinfection. Statistically significant differences ($P < 0.001$) are indicated above the bars (two asterisks) and were determined by performing a two-way analysis of variance for the relative fluorescence units using GraphPad Prism software ($n = 5$).

Guinea pig infection. Six-week-old female Hartley guinea pigs were inoculated intraperitoneally (i.p.) with 1,000 PFU of either *R. rickettsii* Sheila Smith or *R. rickettsii* Iowa or an equivalent amount of formaldehyde-fixed *R. rickettsii* strain R, and their temperatures were monitored rectally for 14 days after infection. For immunization experiments guinea pigs were challenged i.p. with 1,000 PFU of *R. rickettsii* Sheila Smith 30 days after the initial infection. Blood was drawn from the guinea pigs at zero time and 30 days after infection, and antibody titers were determined by microimmunofluorescence.

LDH release assay. Monolayers of Vero cells in 96-well plates were infected with either *R. rickettsii* strain R or *R. rickettsii* Iowa at a multiplicity of infection (MOI) of 0.1. The percentage of total lactate dehydrogenase (LDH) released into the medium by *R. rickettsii* was determined using a CytoTox-ONE homogeneous membrane integrity assay kit (Promega) on days 1, 3, 4, 5, 6, and 7 as described by the manufacturer.

Western blotting. *R. rickettsii* strain R and *R. rickettsii* Iowa (2×10^8 cells) were resuspended in 100 μl of Laemmli buffer. Protein from equal volumes of solubilized cells was separated by electrophoresis on a 10% sodium dodecyl sulfate-polyacrylamide gel for 1 h at 150 V. Proteins were transferred at 100 V for 1 h to a nitrocellulose membrane, and rOmpA was detected using anti-rOmpA monoclonal antibodies 13-3 and 13-5 (2).

Dual fluorescence staining of rickettsiae and F-actin. *R. rickettsii* strain R suspended in Hanks balanced salt solution was used to infect monolayers of Vero cells on 12-mm coverslips at an MOI of 5 for 30 min. Fresh M199 medium plus 2% fetal bovine serum was then added, and incubation was continued at 34°C. Cells were fixed in 4% paraformaldehyde, 25 mM $NaPO_4$, 150 mM NaCl for

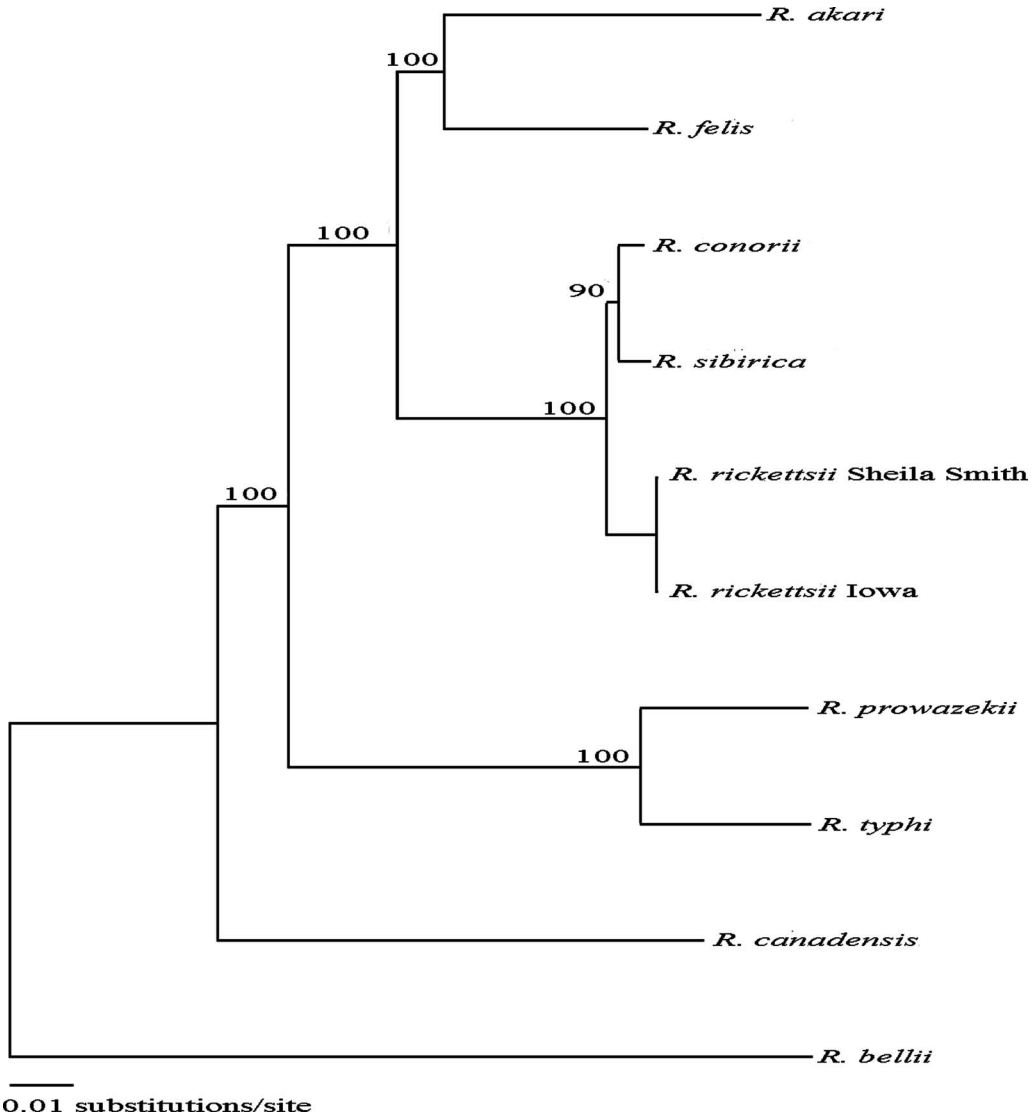

FIG. 2. Phylogram showing that *R. rickettsii* Sheila Smith and *R. rickettsii* Iowa are closely related. The following concatenated sequences of *Rickettsia* species were used for the analysis: *glt*, *gyrB*, *ompB*, *recA*, and *sca4*. *R. bellii* was used as an outgroup. The tree was constructed with Clustal V and the neighbor-joining method with 10,000 bootstrap replicates. A number at a node is the percentage of bootstrap replicates that supported the branching pattern to the right. The scale bar for the branch lengths indicates the number of substitutions per site.

20 min, which was followed by three washes in phosphate-buffered saline (PBS) (25 mM $NaPO_4$, 150 mM NaCl). Cells were treated with 0.1% Triton X-100 in PBS for 4 min to permeabilize the plasma membrane. Cells were then washed three times with PBS. *R. rickettsii* strain R and *R. rickettsii* Iowa were first incubated with anti-OmpB monoclonal antibody 13-2 (2) and stained with anti-mouse immunoglobulin Alexa 488. After the rickettsiae were stained, F-actin was labeled with rhodamine phalloidin (10 U/ml). Cells were then washed three times with PBS and viewed with a Zeiss LSM 510 META confocal microscope.

Microarray analysis. Monolayers of Vero cells in T25 tissue culture flasks were infected with *R. rickettsii* Iowa and *R. rickettsii* strain R at an MOI of 0.05 and incubated for 3 days. To harvest RNA, the medium was removed and 1 ml of Trizol (Invitrogen) was added to each T25 flask for RNA extraction. Samples were transferred to 1.5-ml Eppendorf tubes, and 200 μl of chloroform was added. The samples were then centrifuged at 20,000 × *g* for 15 min. The aqueous phase (~750 μl) was removed and dried to obtain a volume of ~200 μl, and the RNA was purified using an RNeasy 96 kit (Qiagen) according to the manufacturer's instructions. The purified RNA was subjected to two rounds of DNAfree (Ambion) treatment and repurified using the RNeasy 96 kit. SuperScript III (Invitrogen) was used to make cDNA from 10 μg of purified RNA. Five micrograms of cDNA was digested with 0.005 U DNase I (Roche) and labeled using a BioArray terminal labeling kit with biotin-ddUTP (Enzo) according to the manufacturer's instructions. Biotinylated *R. rickettsii* cDNA was hybridized to a custom Affymetrix GeneChip (no. 3 RMLchip3a520351F) containing 1,991 probe sets from two different *R. rickettsii* strains (Sheila Smith and Iowa). Hybridization was performed at 40°C, while scanning and analyses were performed as previously described (32).

TABLE 1. Genomic statistics for *R. rickettsii* Sheila Smith and *R. rickettsii* Iowa

Strain	Total no. of bases sequenced	Total no. of G+C bases	G+C content (%)
Smith	1,257,710	408,346	32.47
Iowa	1,268,175	411,482	32.45

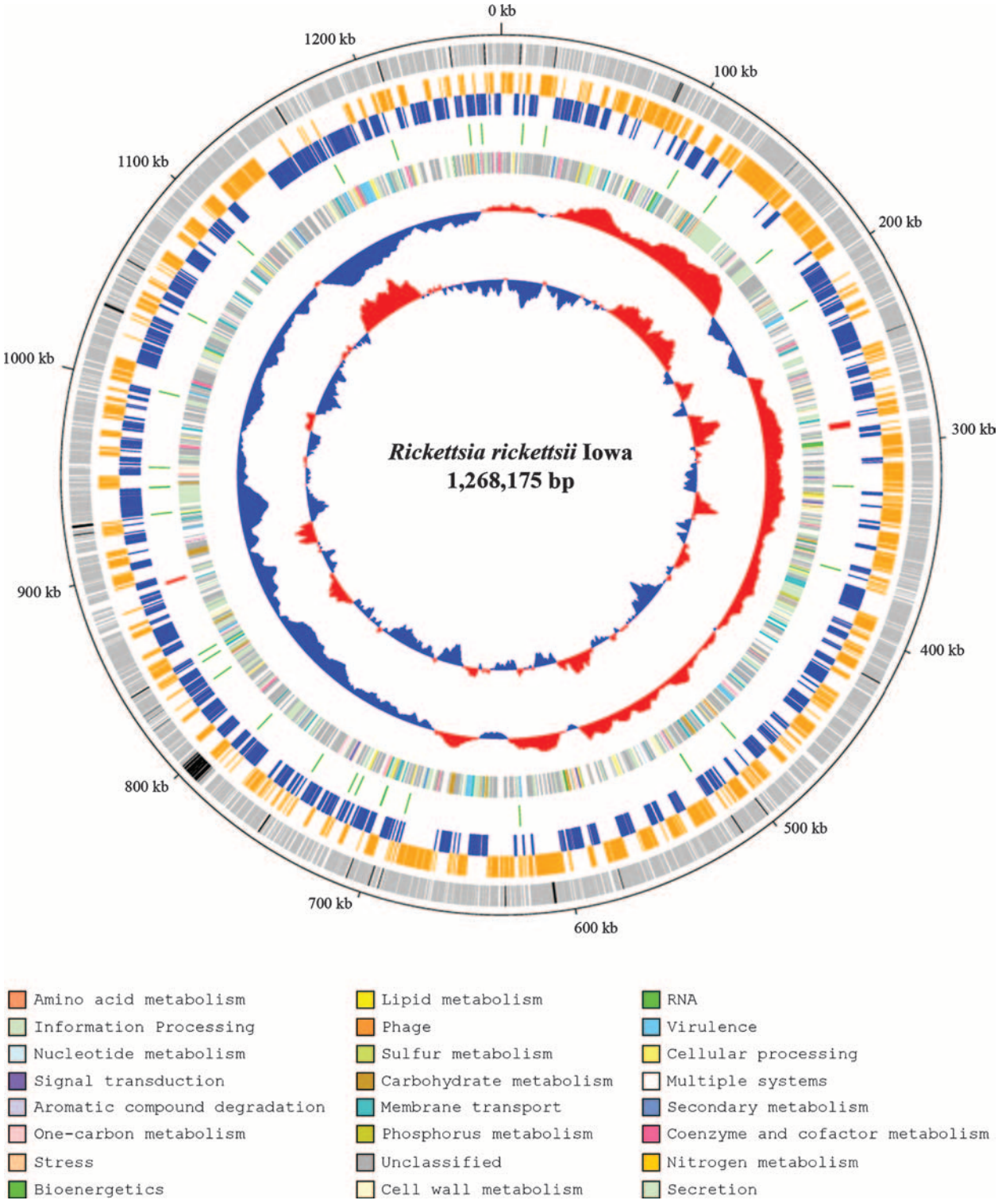

FIG. 3. Circular diagram of the *R. rickettsii* Iowa genome. The outer circle is an alignment of each ORF identified in *R. rickettsii* Iowa compared to ORFs in *R. rickettsii* Sheila Smith. ORFs that share an E value cutoff of $\geq 1e10^{-10}$ are gray, while ORFs that lack *R. rickettsii* Iowa homology are black. The second and third circles are the predicted ORFs in *R. rickettsii* Iowa and the strand of DNA that encodes them (orange, positive strand; blue, negative strand). The fourth circle shows the locations of RNA-encoding regions; tRNAs are green, and rRNAs are red. The fifth circle shows all of the ORFs color coded by functional category, as indicated at the bottom. The sixth circle shows GC skew, and the seventh circle shows the G+C content with a sliding 20-kb window.

Microarray data accession number. Microarray data are posted on the Gene Expression Omnibus website (www.ncbi.nlm.gov/geo/) under accession number GSE8041.

RESULTS

***R. rickettsii* Iowa is avirulent and defective in cell lysis.** A clonal isolate of *R. rickettsii* Iowa was derived from a single plaque from a high-egg-passage stock (EP 271). To confirm that this isolate was avirulent, its ability to induce fever in female Hartley guinea pigs was compared to that of *R. rickettsii* Sheila Smith (Fig. 1A). *R. rickettsii* Iowa did not induce fever in female Hartley guinea pigs when 1,000 PFU was injected i.p. In contrast, *R. rickettsii* Sheila Smith induced marked fever starting on day 3 after infection, which continued until day 12. Guinea pigs infected with *R. rickettsii* Sheila Smith or *R. rickettsii* Iowa also developed high titers of antibodies (see below). Animals inoculated with an equivalent amount of formalin-killed rickettsiae did not develop fever or detectable titers of antibodies (see below), suggesting that replication of *R. rickettsii* Iowa was required to produce sufficient antigenic stimulus to induce significant antibody production. These data suggest that *R. rickettsii* Iowa infected but was unable to induce fever in female Hartley guinea pigs.

R. rickettsii Iowa has previously been shown to form opaque plaques, compared to the clear plaques produced by *R. rickettsii* strain R (18). This observation suggests that *R. rickettsii* Iowa is deficient in host cell lysis. To quantify the abilities of *R. rickettsii* strain R and *R. rickettsii* Iowa to lyse cells, release of LDH into the media was measured during infection (11) (Fig. 1B). Consistent with the observed plaque morphologies, the results showed that there was a statistically significant difference ($P < 0.001$) in the amounts of LDH released on days 6 and 7 postinfection by *R. rickettsii* strain R and *R. rickettsii* Iowa. The data indicate that there is a correlation between the ability of *R. rickettsii* to lyse host cells and the virulence of the organism.

Both *R. rickettsii* strain R and *R. rickettsii* Sheila Smith were isolated in western Montana, and they have similar virulence traits (1, 3, 4, 11). We further showed that *R. rickettsii* strain R and *R. rickettsii* Sheila Smith have no major differences in gene content by hybridizing genomic DNA from the two strains to microarrays containing both the Sheila Smith and Iowa genomes (data not shown). We used both *R. rickettsii* strain R and *R. rickettsii* Sheila Smith as representative western Montana strains throughout this study based on their phenotypic and genetic similarities.

Genomic characterization. To determine which genes may be involved in the virulence of *R. rickettsii*, the genomic sequence of *R. rickettsii* Iowa was determined and compared to that of *R. rickettsii* Sheila Smith. The genome of *R. rickettsii* Iowa consists of a single circular chromosome predicted to contain 1,567 ORFs. A high degree of sequence identity (96.6%) was observed for the two genomes. When deleted regions are discounted, the identity between the two genomes is ~ 99%. A multilocus sequence alignment confirmed that *R. rickettsii* Iowa and *R. rickettsii* Sheila Smith are closely related (Fig. 2), and the results were similar to previously published results (14). Genomic features of both strains are listed in Table 1, and a detailed comparison of the two genomes is shown in Fig. 3. Despite the high degree of identity between the two genomes, a number of strain-specific insertions, deletions, and SNPs were identified.

TABLE 2. Deletions in *R. rickettsii* Sheila Smith compared to *R. rickettsii* Iowa

Location	Size (bp)	Result
Rick02001026/ Rick02001027	10,585	Deletes RrIowa0964-RrIowa0983
Rick02000769/ Rick02000770	857	Deletes RrIowa0725-RrIowa0726
Rick02001265	609	Truncated by 93 bp
Rick02000149	72	In-frame deletion
Rick02001088	27	Truncated by 36 bp
Rick02000842	23	Frameshift adds 85 bp
Rick02000149	16	In-frame deletion
Rick02001130	16	Frameshift adds 209 bp
Rick02001362	6	In-frame deletion
Rick02001493	4	Truncated by 40 bp
Rick02000149	3	In-frame deletion
Rick02001442	3	In-frame deletion
Rick02001549	3	In-frame deletion
Rick02000797	2	Frameshift adds 268 bp
Rick02000821	2	Truncated by 97 bp
Rick02000195	1	Frameshift adds 7 bp
Rick02000634	1	Frameshift adds 236 bp
Rick02000747	1	Frameshift adds 25 bp
Rick02000852	1	Frameshift adds 18 bp
Rick02000960	1	Frameshift adds 144 bp
Rick02001268	1	Frameshift adds 91 bp
Rick02001323	1	Frameshift adds 159 bp
Rick02001512	1	Truncated by 312 bp

Deletion analysis. To determine genomic differences between *R. rickettsii* Iowa and *R. rickettsii* Sheila Smith, a whole-genome alignment was prepared and manually inspected for the presence of small deletions. A total of 143 deletions ranging from 1 bp to more than 10,000 bp were found for the two genomes. Forty-seven of these deletions were located within predicted ORFs; 23 of them were deletions in *R. rickettsii* Sheila Smith compared to strain Iowa (Table 2), and 24 were deletions in *R. rickettsii* Iowa compared to strain Sheila Smith (Table 3). These deletions included gene truncations, in-frame deletions, frameshifts, and total gene deletions.

Evidence for genomic reduction. The largest difference between *R. rickettsii* Iowa and *R. rickettsii* Sheila Smith is a ~10-kb deletion from *R. rickettsii* Sheila Smith. Genes in this region in *R. rickettsii* Iowa appear to be undergoing degradation. An alignment of this region with the sequences of other rickettsial species suggested that some of the genes in this region have been fragmented or lost in some species and appear to be intact in other species (Fig. 4). Reductive evolution suggests that these genes may not be required for survival within the host or vector (5). One of the genes in this region encodes a potential integral membrane protein, and another encodes a protein with homology to enoyl-coenzyme A hydratase. Because enoyl-coenzyme A hydratase has been associated with pathways involved in processes such as fatty acid biosynthesis, fatty acid metabolism, amino acid degradation, and metabolism, it is reasonable to speculate that *R. rickettsii* may not require the function of this gene to survive within its host cell. Therefore, deletion of this ~10-kb region in *R. rickettsii* Iowa may represent an intermediate step in the process of

TABLE 3. Deletions in *R. rickettsii* Iowa compared to *R. rickettsii* Sheila Smith

Location	Size (bp)	Result
RrIowa1459	891	In-frame deletion
RrIowa1088	590	Truncated by 582 bp
RrIowa1055	39	In-frame deletion
RrIowa1479	32	Truncated by 121 bp
RrIowa0663	14	Frameshift adds 437 bp
RrIowa1265	13	Truncated by 69 bp
RrIowa0440	11	Frameshift adds 155 bp
RrIowa0830	11	Frameshift adds 8 bp
RrIowa0380	5	Frameshift adds 225 bp
RrIowa0280	4	Frameshift adds 81 bp
RrIowa0664	4	Truncated by 341 bp
RrIowa0756	4	Truncated by 50 bp
RrIowa0664	3	In-frame deletion
RrIowa0855	2	Truncated by 133 bp
RrIowa0871	2	Truncated by 42 bp
RrIowa0517	1	Frameshift adds 51 bp
RrIowa0622	1	Changes start codon to GTG from TTG
RrIowa0623	1	Truncated by 116 bp
RrIowa0630	1	Frameshift adds 130 bp
RrIowa0792	1	Truncated by 457 bp
RrIowa0867	1	Frameshift adds 481 bp
RrIowa0917	1	Truncated by 38 bp
RrIowa1113	1	Frameshift adds 199 bp
RrIowa1460	1	Truncated by 4,536 bp

genomic reduction. There are also a number of genes shared by the two genomes that appear to be fragmenting and possibly represent a snapshot of genomic reduction. Genomic reduction has been hypothesized and described previously for a comparison of the genomes of *R. conorii* and *R. prowazekii* (26). Here we identified an apparently similar process in the genomes of strains belonging to the same species, *R. rickettsii*.

rOmpA is disrupted in *R. rickettsii* Iowa. Comparison of *R. rickettsii* Iowa to *R. rickettsii* Sheila Smith revealed two deletions in *rompA* (RrIowa1460), a 1-bp deletion that introduces a stop codon truncating *rompA* 660 bp from the start codon and an 891-bp in-frame deletion located within the repeat region of *rompA* (Fig. 5A). rOmpA is one of the major outer membrane proteins found in *R. rickettsii* and is believed to contribute to virulence (15, 24). To test for the presence of rOmpA in *R. rickettsii* Iowa, Western blotting was performed with purified *R. rickettsii* Iowa and *R. rickettsii* strain R using rOmpA-specific monoclonal antibodies (2) (Fig. 5B). No full-length rOmpA or any truncated rOmpA products were detected in *R. rickettsii* Iowa, whereas a single rOmpA band was detected in *R. rickettsii* strain R. Immunofluorescence confirmed the absence of rOmpA in *R. rickettsii* Iowa (data not shown). These results suggest that the 1-bp deletion causes a truncation in *rompA* as no full-length rOmpA was detected in *R. rickettsii* Iowa, and they suggest that the truncated product was either rapidly degraded or not detected by our monoclonal antibodies.

***R. rickettsii* Iowa has actin-based motility.** *R. rickettsii* relies on actin-based motility to propel itself from one host cell to another. It has recently been suggested that rOmpA may play a role in the actin-based motility of rickettsiae (7). RickA has previously been shown to be involved in the actin-based motility of rickettsiae (16, 22), but there is a 39-bp in-frame deletion in the gene in *R. rickettsii* Iowa. Taken together, these two observations suggested that *R. rickettsii* Iowa may have a defect in its actin-based motility. To determine if the mutations affected the actin-based motility of *R. rickettsii* Iowa, we confirmed that *R. rickettsii* Iowa was able to form actin tails during infection of Vero cells. On day 1 after infection no difference in the abilities of *R. rickettsii* Iowa and *R. rickettsii* strain R to form actin tails was observed (Fig. 6). The ability to form actin tails was also investigated on days 2 and 3 postinfection, and no difference was observed (data not shown). Furthermore, the plaque sizes were indistinguishable, suggesting that the rates of cell-to-cell spread were similar (18). These data are consistent with previous data (20) and suggest that the mutation in rOmpA and the in-frame deletion in RickA do not affect the ability of *R. rickettsii* Iowa to form actin tails.

SNP analysis. To further analyze the genomic differences between *R. rickettsii* Sheila Smith and *R. rickettsii* Iowa, SNPs were identified. When *R. rickettsii* Iowa was compared to *R. rickettsii* Sheila Smith, only 492 SNPs were identified in the two genomes. Of the 492 SNPs, 5 localized to RNA-encoding regions, 148 were located in intergenic regions, and 339 were found in predicted ORFs. For the ORFs identified as ORFs containing SNPs, only 188 SNPs resulted in nonsynonymous amino acid substitutions. Figure 7 shows the number of SNPs per ORF in the 1,302 ORFs shared by *R. rickettsii* Iowa and *R. rickettsii* Sheila Smith. Of the ORFs containing SNPs, only six contain more than two nonsynonymous SNPs (Table 4). One of the genes with multiple SNPs (four SNPs) is *rompB*, which has previously been shown to be defective in processing in *R. rickettsii* Iowa (18). This suggests that one or more of the SNPs could be involved in interfering with the ability of the organism to process rOmpB.

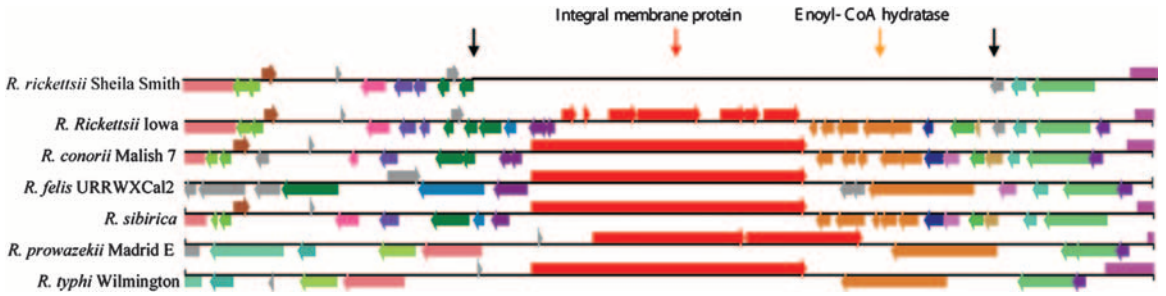

FIG. 4. Evidence of genomic reduction in *R. rickettsii* Sheila Smith: graphic representation of the genomic region deleted from *R. rickettsii* Sheila Smith. The region deleted from *R. rickettsii* Sheila Smith is indicated by black arrows. CoA, coenzyme A.

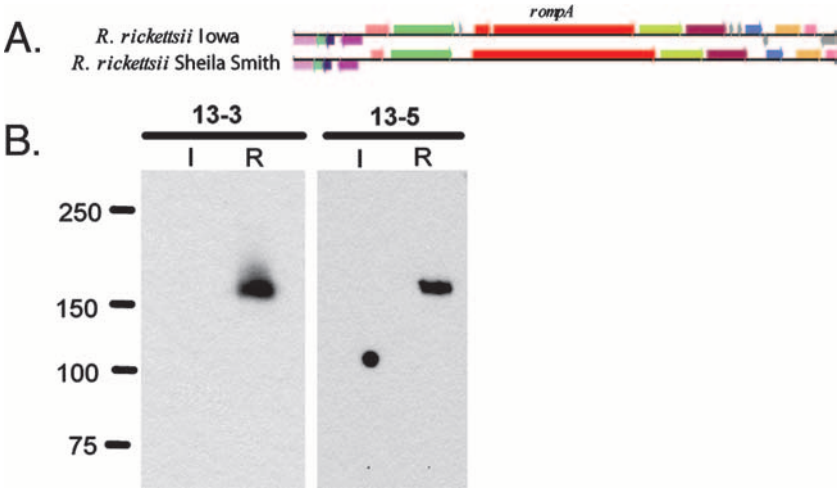

FIG. 5. rOmpA is disrupted in *R. rickettsii* Iowa. (A) Graphic representation of the disrupted *rompA* gene in *R. rickettsii* Iowa. (B) Equal amounts of *R. rickettsii* Iowa (lanes I) and *R. rickettsii* strain R (lanes R) were loaded and run on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and transferred to a nitrocellulose membrane. rOmpA was detected using monoclonal antibodies 13-3 and 13-5 (2).

Expression analysis of *R. rickettsii* Iowa and *R. rickettsii* strain R. Of the 143 deletions and 492 SNPs found in *R. rickettsii* Iowa and *R. rickettsii* Sheila Smith, 96 deletions and 148 SNPs were located in intergenic regions. To study the effect of both intergenic and intragenic mutations on gene expression, custom Affymetrix GeneChips were used to determine gene expression differences between *R. rickettsii* Iowa and *R. rickettsii* strain R. To determine differences in gene expression, *R. rickettsii* Iowa and *R. rickettsii* strain R were grown in Vero cells for 3 days (~2.0×10^6 PFU) before RNA was harvested and hybridized to microarrays. The data indicate that the expression of only four genes was significantly different in *R. rickettsii* strain R and *R. rickettsii* Iowa. Thus, the majority of the genetic differences do not affect gene expression (Table 5). Of the four genes that differ in expression, two have differences in the predicted promoter regions and two have 1-bp deletions that result in truncation of the gene.

***R. rickettsii* Iowa provides protection against *R. rickettsii* Sheila Smith challenge.** To determine if guinea pigs infected with *R. rickettsii* Iowa developed immunity against subsequent challenge with virulent *R. rickettsii*, naïve guinea pigs and guinea pigs that had previously been infected with 1,000 PFU of *R. rickettsii* Iowa or Sheila Smith and vaccinated with equivalent amounts of paraformaldehyde-fixed *R. rickettsii* strain R were challenged 30 days after the primary infection with 1,000 PFU of *R. rickettsii* Sheila Smith. All of the naïve guinea pigs and the guinea pigs inoculated with formalin-killed rickettsiae developed fever after challenge with *R. rickettsii* Sheila Smith, suggesting that the equivalent of 1,000 PFU of formalin-killed rickettsiae was insufficient to elicit protection against challenge with *R. rickettsii* Sheila Smith (Table 6). However, all of the guinea pigs infected with *R. rickettsii* Sheila Smith and four of five guinea pigs infected with *R. rickettsii* Iowa were protected against challenge with *R. rickettsii* Sheila Smith (Table 6). One of the *R. rickettsii* Iowa-vaccinated guinea pigs failed to protect against challenge with *R. rickettsii* Sheila Smith because it did

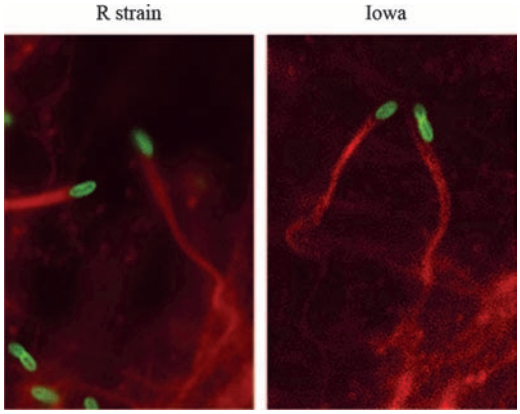

FIG. 6. *R. rickettsii* Iowa shows normal actin tail formation. Monolayers of Vero cells were infected with either *R. rickettsii* Iowa or *R. rickettsii* strain R and allowed to grow for 1 day. *R. rickettsii* was detected using monoclonal antibody 13-2 (1/100), while F-actin was labeled with rhodamine phalloidin (10 U/ml).

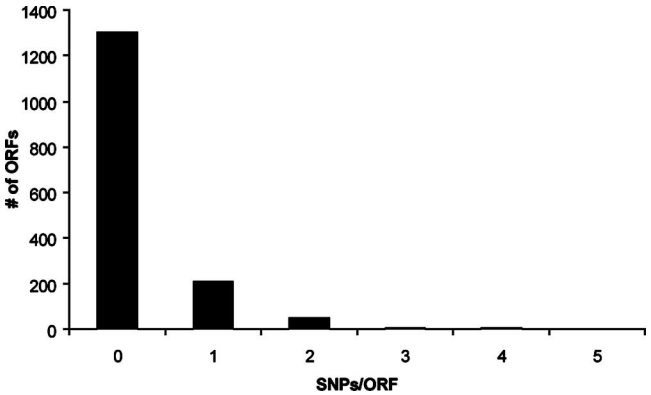

FIG. 7. SNPs present in *R. rickettsii* Iowa and *R. rickettsii* Sheila Smith. The numbers of SNPs found in specific ORFs are indicated on the *x* axis. The total numbers of SNPs/ORF are indicated on the *y* axis.

TABLE 4. ORFs containing more that two nonsynonymous SNPs

ORF	No. of SNPs	Function
RrIowa0140	4	Hypothetical autotransporter
RrIowa0569	4	NADH-quinone oxidoreductase chain A
RrIowa0804	4	Biotin operon repressor
RrIowa1264	4	rOmpB
RrIowa0300	3	Transpeptidase-transglycosylase PBP 1C
RrIowa0177	3	Channel protein VirB6

TABLE 6. *R. rickettsii* Iowa protects against challenge with *R. rickettsii* Sheila Smith

Infection	Challenge	No. of guinea pigs with fever/total no.	Antibody titer (range)[a]
Sheila Smith	Sheila Smith	0/5	10,880 (3,200–12,800)
Iowa	Sheila Smith	1/5	3,072 (0–12,800)
Formalin killed	Sheila Smith	4/4	0 (0)
Naïve	Sheila Smith	5/5	0 (0)

[a] Antibody titers were determined 30 days after vaccination.

not develop a detectable antibody response. These results suggest that *R. rickettsii* Iowa is able to replicate after infection and stimulate an immune response that protects against challenge with *R. rickettsii* Sheila Smith.

DISCUSSION

Strains of *R. rickettsii* vary dramatically in their virulence in animal model systems and in the severity of human disease that they cause (1, 3, 4, 11, 30). The obligate intracellular lifestyle of rickettsiae and the lack of tractable genetic systems make it difficult to identify genes involved in virulence. With the completed sequences of multiple rickettsial species, it has become possible to investigate differences between virulent and avirulent strains of rickettsiae through comparative genomics. To determine genomic differences between virulent and avirulent strains of *R. rickettsii*, the sequence of the avirulent strain *R. rickettsii* Iowa was determined and compared to that of the virulent strain *R. rickettsii* Sheila Smith.

Both multilocus sequence alignment and genomic comparison of *R. rickettsii* Iowa and *R. rickettsii* Sheila Smith confirmed that these two strains are very closely related. Only two major lesions resulting in gene deletions were found in the two strains (Table 2). Both of these deletions are in *R. rickettsii* Sheila Smith, as well as the virulent R strain of *R. rickettsii*, based on microarray analysis of the two genomes. However, the genes in both of the regions appear to be undergoing degradation in *R. rickettsii* Iowa. This indicates that these regions may not be essential for survival of the organism in either the host or the vector and that in virulent *R. rickettsii* these genes have been deleted. Genomic reduction in rickettsiae has been described previously (26), and the deletion of genes revealed by a comparison of *R. rickettsii* Sheila Smith and *R. rickettsii* Iowa is consistent with this hypothesis.

A 1-bp deletion in the 5′ end of *R. rickettsii* Iowa *rompA* introduces a stop codon at amino acid 184 resulting in a premature stop in translation. As a result of this 1-bp deletion, *R. rickettsii* Iowa does not produce rOmpA. rOmpA has previously been implicated in attachment of *R. rickettsii* to host cells (24). However, no change in the efficiency of plating or growth rate of *R. rickettsii* Iowa in the Vero or ISE6 cell line was observed compared to the results for virulent *R. rickettsii* (data not shown). This suggests that rOmpA is not absolutely required for adherence and entry of *R. rickettsii* in vitro. rOmpB, which is defective in processing but is still present in the outer membrane of *R. rickettsii* Iowa, has been shown to interact with host cell protein KU70 to promote entry of *R. rickettsii* (24a). This interaction, in the absence of rOmpA, may be sufficient for adherence and uptake of *R. rickettsii* Iowa in vitro. However, the possibility that interactions mediated by rOmpA or rOmpB do play a significant role in infection by *R. rickettsii* cannot be ruled out as *R. rickettsii* Iowa was unable to induce fever in guinea pigs.

As previously shown (18), *R. rickettsii* Iowa is deficient in the processing of rOmpB from the 168-kDa precursor form into the 120- and 32-kDa forms of the protein. In a comparison of the genomes of *R. rickettsii* Sheila Smith and *R. rickettsii* Iowa, there were no obvious lesions that accounted for the defect in processing. A direct sequence alignment of the rOmpB proteins from *R. rickettsii* Iowa and *R. rickettsii* Sheila Smith showed that there were four nonsynonymous changes in rOmpB from *R. rickettsii* Iowa. It is possible that one or more of these changes disrupt the processing of rOmpB. A more detailed site-specific mutation strategy using a heterologous *Escherichia coli* expression system may prove to be essential for determining if any of these changes contribute to the defect in processing of rOmpB in *R. rickettsii* Iowa.

Both intergenic and intragenic deletions were found when *R. rickettsii* Iowa and *R. rickettsii* Sheila Smith were compared. There were also a number of SNPs in the two strains that were located in intergenic regions. Any of these differences could lead to altered gene expression. In a comparison of the gene expression levels in *R. rickettsii* Iowa and *R. rickettsii* strain R, we determined that four genes exhibited significant changes in expression (not including deleted genes). One of these genes was *rompA*, which has been divided into two ORFs because of a 1-bp deletion. Microarray analysis showed that the expression of the 3′ ORF of *rompA* (RrIowa1459) in *R. rickettsii* Iowa was reduced compared to the expression of *R. rickettsii* strain R *rompA*. We hypothesize that this could be due to stalling of the ribosomes and disengagement of mRNA at the premature stop codon, thereby causing destabilization of the 3′ half of the message. A result of this destabilization may be an increased rate of degradation of the 3′ region of *rompA*, decreasing the signal for *rompA* (RrIowa1459) in *R. rickettsii* Iowa. A second gene in *R. rickettsii* Iowa (RrIowa0791) that is split by a pre-

TABLE 5. Expression differences between *R. rickettsii* Iowa and *R. rickettsii* strain R

Gene	Fold change	Mutation
RrIowa1459	−2.7	1-bp deletion introduces stop codon
RrIowa1486	−3.1	Contains two SNPs in potential promoter region
RrIowa0791	−4.3	1-bp deletion introduces stop codon
RrIowa0614	2.5	14-bp deletion in potential promoter region

mature stop codon shows a similar effect, with the 3′ end of the gene displaying down regulation compared to the *R. rickettsii* strain R gene. However, most of the genomic differences between *R. rickettsii* Iowa and *R. rickettsii* Sheila Smith do not appear to affect gene expression.

It is apparent that in addition to the processing defect in rOmpB, *R. rickettsii* Iowa is also deficient in rOmpA. This leads to defects in two of the major factors thought to be involved in the virulence of *R. rickettsii*. In the absence of methods to genetically manipulate rickettsiae, it would be of great interest to isolate clonal variants which are defective in either rOmpB or rOmpA to test the effect of each mutation on virulence individually. As shown in Tables 2 and 3, the differences between *R. rickettsii* Iowa and *R. rickettsii* Sheila Smith are not limited to rOmpB and rOmpA. However, without a good genetic system available, comparison of multiple genomes of virulent and avirulent isolates may prove to be the most expedient method to identify bacterial factors that play a role in the pathogenesis of *R. rickettsii*.

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